1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of CpG O-ODN. The maximum levels of IL-6 induced by bacterial DNA and CpG ODN were 1-1.5 ng/ml and 2-4 ng/ml respectively. These levels were significantly greater than those seen-after stimulation by LPS (0.35 ng/ml) (Fig. 1A). To evaluate whether CpG ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. CpG S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as CpG O-ODN while non-CpG S-ODN failed to induce IL-6 (Fig. 1C). CpG S-ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicted that the nuclease-resistant DNA backbone modification retains the sequence specific ability of CpG DNA to induce IL-6 secretion and that CpG S-ODN are more than 80-fold more potent than CpG O-ODN in this assay system.

Induction of Murine IL-6 murine by CpG DNA in vivo

To evaluate the ability of bacterial DNA and CpG S-ODN to induce Il-6 secretion *in vivo*, BALB/c mice were injected iv. with 100 μg of *E. coli* DNA, calf thymus DNA, or CpG or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the *E. coli* DNA injected group as approximately 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). CpG S-ODN also induced IL-6 secretion *in vivo*. The IL-6 level in the sera from CpG S-ODN injected groups was approximately 20 ng/ml. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated group (Table 4).

Table 4. Secretion of Murine IL-6 induced by CpG DNA stimulation *in vivo*.

Stimulant	IL-6 (pg/ml)
PBS	< 50
E. coli DNA	13858 ± 3143
Calf Thymus DNA	< 50
CpG S-ODN	20715 ± 606
non-CpG S-ODN	< 50

Mice (2 mice/group) were i.v. injected with 100 μl of PBS, 200 μl of *E. coli* DNA or calf thymus DNA, or 500 μg of CpG S-ODN or non-CpG control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the CpG S-ODN is 5'GCATGACGTTGAGCT3' (SEQ. ID. No:6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. No:4). Note that although there is a CpG in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean ± SD of duplicates. The experiment was done at least twice with similar results.

results are shown in Table 11.

Effective ODNs began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal CpG motifs (e.g., ODN 1840) are generally less potent stimulators than those in which a GTCGCT (SEQ. ID. NO: 58) motif immediately follows the 5' TC (e.g., ODN 1967 and 1968). ODN 1968, which has a second GTCGTT SEQ. ID. NO: 57) motif in its 3' half, was consistently more stimulatory than ODN 1967, which lacks this second motif. ODN 1967, however, was slightly more potent than ODN 1968 in experiments 1 and 3, but not in experiment 2. ODN 2005, which has a third GTCGTT (SEQ. ID. NO. 57) motif, inducing slightly higher NK activity on average than 1968. However, ODN 2006, in which the spacing between the GTCGTT (SEQ. ID. NO: 57) motifs was increased by the addition of two Ts between each motif, was superior to ODN 2005 and to ODN 2007, in which only one of the motifs had the additional of the spacing two Ts. The minimal acceptable spacing between CpG motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN 2015). Surprisingly, joining two GTCGTT (SEQ. ID. NO: 57) motifs end to end with a 5' T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive CpG dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGTT; SEQ. ID. NO: 113). It should also be noted that ODNs containing no CpG (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contents (e.g., ODN 2010) had no stimulatory effect on NK activation.

Table 10

ODN	Sequence (5'-3')	LU	
cells alone		0.01	
1754	ACCATGGACGATCTGTTTCCCCTC	0.02	SEQ ID NO:59
1758	TCTCCCAGCGTGCGCCAT	0.05	SEQ ID NO:45
1761	TACCGCGTGCGACCCTCT	0.05	SEQ ID NO:60
1776	ACCATGGACGAACTGTTTCCCCTC	0.03	SEQ ID NO:61
1777	ACCATGGACGAGCTGTTTCCCCTC	0.05	SEQ ID NO:62
1778	ACCATGGACGACCTGTTTCCCCTC	0.01	SEQ ID NO:63
1779	ACCATGGACGTACTGTTTCCCCTC	0.02	SEQ ID NO:64
1780	ACCATGGACGGTCTGTTTCCCCTC	0.29	SEQ ID NO:65
1781	ACCATGGACGTTCTGTTTCCCCTC	0.38	SEQ ID NO:66
1823	GCATGACGTTGAGCT	0.08	SEQ ID NO:6
1824	CACGTTGAGGGGCAT	0.01	SEQ ID NO:67
1825	CTGCTGAGACTGGAG	0.01	SEQ ID NO:68
1828	TCAGCGTGCGCC	0.01	SEQ ID NO:69
1829	ATGACGTTCCTGACGTT	0.42	SEQ ID NO:70
1830[2]	RANDOM SEQUENCE	0.25	
1834	TCTCCCAGCGGGCGCAT	0.00	SEQ ID NO:71
1836	TCTCCCAGCGCGCGCCAT	0.46	SEQ ID NO:72
1840	TCCATGTCGTTCCTGTCGTT	2.70	SEQ ID NO:73
1841	TCCATAGCGTTCCTAGCGTT	1.45	SEQ ID NO:74
1842	TCGTCGCTGTCTCCGCTTCTT	0.06	SEQ ID NO:75
1851	TCCTGACGTTCCTGACGTT	2.32	SEQ ID NO:76